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PRECLINICAL RESEARCH

Lipopolysaccharide Activates Calcineurin in Ventricular Myocytes

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Objectives We investigated whether lipopolysaccharide (LPS), a proximate cause of inflammation, activates calcineurin in cardiac myocytes and if calcineurin regulates apoptosis in this setting.

Background Calcineurin regulates myocardial growth and hypertrophy, but its role in inflammation is unknown. Calcineurin has proapoptotic or antiapoptotic effects depending on the stimuli.

Methods Calcineurin activity was measured in left ventricular myocytes from adult Sprague Dawley rats. Cardiac apoptosis was measured by terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling staining and caspase-3 activity after in vitro and in vivo exposure to LPS.

Results Lipopolysaccharide increased calcineurin activity in myocytes over 1 to 24 h (t 1/2 = 4.8 h) with an EC_{50} of 0.80 ng/ml LPS ($p < 0.05$, $n = 4$). The LPS (10 ng/ml) effects were mimicked by angiotensin II (Ang II) (100 nmol/l); both increased calcineurin activity and induced apoptosis without additive effects ($p < 0.05$, $n = 5$ to 9). Lipopolysaccharide and/or Ang II effects were prevented by 1 h pre-treatment with an Ang II type 1 receptor blocker (losartan, 1 μmol/l), calcineurin inhibitor (cyclosporin A, 0.5 μmol/l), calcium chelator (1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl) ester, 0.1 μmol/l), or by inhibiting sarcoplasmic reticulum (SR) calcium (Ca)-ATPase (thapsigargin, 1 μmol/l) or SR calcium release channel (ryanodine, 1 μmol/l). Left ventricular apoptosis increased from 4 to 24 h after LPS (1 mg/kg intravenously) in vivo, but not in rats pre-treated with cyclosporin A (20 mg/kg/day subcutaneously) for 3 days ($p < 0.05$, $n = 5$).

Conclusions In cardiac myocytes, LPS activates calcineurin in association with apoptosis by Ang II and SR calcium-dependent mechanisms. This expands the paradigm for cardiac calcineurin to be activated by low levels of LPS in inflammation and chronic conditions (e.g., infections, smoking, and heart failure). (J Am Coll Cardiol 2007;49:491–9)

Calcineurin is an important mediator of cardiac growth and hypertrophy (1,2). The stress signals that activate cardiac calcineurin in physiological and pathophysiological conditions, such as pressure overload and ischemia, have been studied extensively. However, little is known about the role of calcineurin in inflammation. Inflammation contributes to the pathogenesis of atherosclerosis and vascular events (3) and may contribute to the progression of heart failure (4). It is unknown if inflammation activates calcineurin in cardiac myocytes.

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Lipopolysaccharide (LPS) from gram negative bacteria is one of the most common causes of inflammation and the best characterized activator of innate immunity (5,6). Low levels of LPS may mediate vascular inflammation in atherosclerosis (7). Cells sense minute amounts of LPS through Toll-like receptor-4 (TLR-4), an LPS receptor that recognizes pathogen-associated molecular patterns and is required for cell signaling. Cardiac myocytes express TLR-4 (8), although they are not professional cells involved in innate immunity. Studies from this laboratory demonstrated that LPS directly activates cardiac myocytes to depress contractility (9) and induce apoptosis (10), independently of secondary mediators released by non-myocytes. This occurs with low levels of LPS comparable to those found circulating in subacute

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Abbreviations and Acronyms

Ang II = angiotensin II

AT_1 = angiotensin II type 1 receptor

BAPTA-AM = 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester

LPS = lipopolysaccharide

MPT = mitochondrial permeability transition pore

RyR = ryanodine receptor

SR = sarcoplasmic reticulum

TLR-4 = Toll-like receptor-4

TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

and chronic conditions, such as chronic infections, smoking, and heart failure (11–13).

The goal of this study was to determine if LPS activates calcineurin in cardiac myocytes. This would expand the list of known activators of cardiac calcineurin to include inflammation, along with previously established conditions of cardiac growth, hypertrophy, and ischemia-reperfusion. Calcineurin may be activated by LPS in macrophages (14), but it is unknown if LPS activates calcineurin in cardiac myocytes.

Calcineurin is a serine/threonine protein phosphatase that is activated by a sustained increase in calcium. Lipopolysaccharide increases intracellular calcium in cardiac myocytes (15,16). It is unknown if this is sufficient to activate calcineurin, which may depend on specialized pools of calcium at specific intracellular sites (17,18). Several sites for activating calcium have been proposed, but none have been proven experimentally (17). We hypothesized that LPS activates calcineurin with calcium cycling through the sarcoplasmic reticulum (SR). The rationale is that the SR plays a central role for calcium handling and there is a physical and functional association between calcineurin and the SR calcium release channel or ryanodine receptor (RyR) (19,20). The calcineurin inhibitor FK506 targets FK506 binding proteins, which regulate RyR function (21).

A secondary goal was to determine if calcineurin plays a regulatory role to enhance or inhibit cardiac apoptosis induced by LPS. Calcineurin enhances cardiac apoptosis induced by beta-adrenergic stimulation (22), but inhibits apoptosis induced by oxidative stress or ischemia-reperfusion (23,24). Calcineurin can induce or inhibit apoptosis in the same cell depending on the concurrent activation of downstream signaling pathways (25,26). Thus, the effects of calcineurin on apoptosis are stimuli and context-dependent. As a subsidiary goal, we evaluated if calcineurin is activated by the same cell signaling pathway as LPS-induced apoptosis, which we found to be mediated by activating cardiac renin-angiotensin to stimulate angiotensin II type 1 (AT_1) receptors (10,27). Angiotensin II (Ang II) increases calcineurin in cardiac myocytes (28,29).

This study demonstrates that LPS activates calcineurin in association with apoptosis in cardiac myocytes. This occurs with low levels of LPS found in chronic conditions. This provides a unique link between inflammation activated by LPS, and calcineurin, an important signaling pathway for myocardial growth and hypertrophy.

Methods

Experiments were performed in accordance with institutional guidelines and the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cardiac myocyte preparation and protocols. Cardiac myocytes were isolated from adult Sprague Dawley rats (250 to 400 g, either gender), as previously described (10). In brief, rats were anesthetized with 40 mg/kg sodium pentobarbital intraperitoneally. The heart was excised and perfused with 15 to 30 mg/kg of depyrogenated collagenase B and protease containing <0.3 to 0.5 ng/ml LPS (*Limulus* amobocyte lysate test QCL-1000, BioWhittaker, Walkersville, Maryland) (30). Freshly isolated myocytes were plated on dishes pre-coated with laminin in a Dulbeccos modified Eagle's media and stored at 37°C in 5% CO_2 (10).

Myocytes were incubated with LPS (*Escherichia coli* 055, LPS no. B5, lot 2039F, List Biological Laboratories, Campbell, California) and/or Ang II, preceded by 1 h exposure to inhibitors including losartan (AT_1 receptor inhibitor, a kind gift from Merck and DuPont, Rahway, New Jersey), 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM) (calcium chelator), thapsigargin (SR calcium ATPase inhibitor), ryanodine (SR calcium release channel inhibitor, Calbiochem, San Diego, California), cyclosporin A (calcineurin inhibitor), or nicotine (an inhibitor of LPS-induced cardiac apoptosis) (27) (unless otherwise noted, all reagents from Sigma Chemical, St. Louis, Missouri).

Calcineurin and apoptosis assays in cardiac myocytes and left ventricle. Calcineurin assays were performed on cardiac myocytes harvested after 16 h of incubation. After cell lysis, cellular calcineurin (PP2B) phosphatase activity was measured using a BIOMOL GREEN Calcineurin Assay Kit (BIOMOL Research Laboratories, Plymouth Meeting, Pennsylvania). Ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*,-tetraacetic acid (EGTA) was used as the calcineurin inhibitor, and results were expressed as Total − EGTA phosphate nmol/μg total protein. Protein content was determined using a standard colorimetric assay (BCA, Pierce Chemical, Rockford, Illinois). The calcineurin activity data were fit to curves using GraphPad Prism, version 4.0 from GraphPad Software, Inc. (San Diego, California).

Apoptosis was assessed in cardiac myocytes fixed with 4% formalin phosphate-buffered saline after 24 h of incubation. Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed using a CardioTACS In Situ Apoptosis Detection kit (R&D Systems). At least 2,000 cells were scored from each group with the observer blinded to the treatment condition.

In vivo studies were performed in rats injected with LPS (1 mg/kg) or saline into a tail vein (10). After 24 h, the heart was excised and fixed in 3.7% formaldehyde solution for

24 h, and embedded in paraffin. The TUNEL staining was performed on 5-μm cross-section slices to determine the average transmural rate of TUNEL-positive stained cardiomyocyte nuclei per 10^6 nuclei, measured across the left ventricular anterior, midventricular free wall, as previously described (10). The time course for apoptosis was assessed in hearts harvested immediately (time 0), 2, 4, 6, 8, or 24 h after injecting LPS (1 mg/kg intravenously). Caspase-3 activity was measured in the left ventricle with a FluroAce Apopain Assay Kit (Bio-Rad Laboratories, Richmond, California).

Statistical analysis. Results were compared by 1- or 2-way repeated measures analysis of variance (ANOVA) in protocols where myocytes from each rat were subdivided into separate dishes to test individual treatments (n = 1 for each rat heart). Left ventricular data from different animals were analyzed by 1- or 2-way ANOVA. Post-hoc comparisons were performed with Student-Newman-Keuls methods. All results are expressed as mean ± SE. Statistical significance indicates $p < 0.05$. The statistical analyses were performed with SigmaStat Statistical Software, version 2.0 from SPSS Science (Chicago, Illinois).

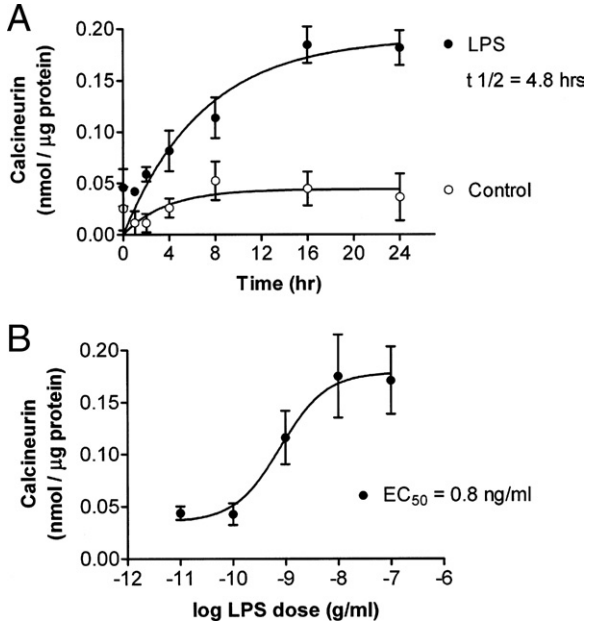

Figure 1 Time Course and LPS Dose Dependence of Calcineurin Activity in Cardiac Myocytes

(A) Calcineurin activity in cardiac myocytes (mean ± SE, n = 4) increased with lipopolysaccharide (LPS) (10 ng/ml) compared with control (vehicle) after 1 to 24 h, with t 1/2 of 4.8 h ($p < 0.05$). **(B)** Lipopolysaccharide dose-calcineurin activity relationship (mean ± SE, n = 4). After 16 h incubation with LPS, calcineurin activity increased in cardiac myocytes with an EC_{50} of 0.80 ng/ml LPS ($p < 0.05$).

Results

LPS increases calcineurin activity in cardiac myocytes. Cardiac myocytes were incubated with LPS (10 ng/ml) or vehicle. Figure 1A shows time-calcineurin activity data (mean with SE bars, n = 4 for each treatment at each time point) fit to a 1-phase exponential curve. After 1, 2, 4, 8, 16, and 24 h, there was a significant increase in calcineurin activity with LPS, but not in vehicle-treated myocytes ($p < 0.05$, 2-way repeated measures ANOVA, $p < 0.001$ for interaction between LPS and time). Although the sample size was small, when the repeated measures ANOVA was performed using the conservative Greenhouse-Geisser adjustment, the interaction between LPS and time remained significant ($p = 0.013$). Calcineurin activity increased with a half-time of 4.8 h after LPS, with a maximum response of 0.19 nmol/μg protein.

The LPS dose-calcineurin activity relationship was determined in cardiac myocytes incubated with 0.01 ng/ml (10^{-11} g/ml) to 100 ng/ml (10^{-7} g/ml) LPS for 16 h. Figure 1B shows data fit to a sigmoidal curve (n = 4 each dose, mean with SE bars). There was higher calcineurin activity with 1, 10, and 100 ng/ml LPS compared with 0.01 or 0.1 ng/ml LPS ($p < 0.05$, 1-way repeated measures ANOVA). Calcineurin activity increased with an EC_{50} of 0.80 ng/ml LPS.

Cell signaling pathways for LPS activation of calcineurin. It was determined if LPS activation of calcineurin involves the same AngII and AT_1 receptor-mediated pathways as LPS-induced cardiac apoptosis (10). Cardiac myocytes were incubated for 16 h with or without LPS (10 ng/ml) and/or Ang II (100 nmol/l). This also was performed in myocytes pre-treated for 1 h with losartan (1 μmol/l). Figure 2 shows that both LPS and Ang II increased calcineurin activity without additive effects ($p < 0.001$, 1-way repeated measures ANOVA, n = 6). The calcineurin activity with LPS + Ang II combined was less than with Ang II alone ($p < 0.05$), but did not differ compared with LPS alone. Neither LPS nor Ang II increased calcineurin activity in the presence of losartan.

Cyclosporin A is a potent and specific inhibitor of calcineurin (31). Calcineurin is a calcium-dependent phosphatase that is inhibited by the calcium chelator BAPTA-AM. To evaluate if the source of calcium for activating calcineurin cycles through the SR, thapsigargin, a Ca-ATPase inhibitor, was used to inhibit SR calcium re-uptake. Myocytes were incubated with vehicle (control) or LPS (10 ng/ml) for 16 h, with or without 1 h of pre-treatment with inhibitors. Figure 3A demonstrates that LPS increased calcineurin activity, which was blocked by either cyclosporin A (0.5 μmol/l), BAPTA-AM (0.1 μmol/l), or thapsigargin (1 μmol/l) ($p < 0.05$, 1-way repeated measures ANOVA, n = 5).

To confirm an SR role, SR calcium cycling was inhibited at a different site using ryanodine, an inhibitor of the SR calcium release channel. Cardiac myocytes were incubated with vehicle (control) or LPS (10 ng/ml) for 16 h, with or

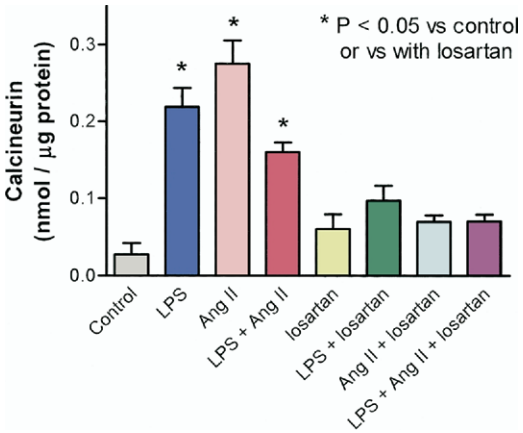

Figure 2 **Calcineurin Activity in Cardiac Myocytes With LPS and/or Ang II Inhibited by Losartan**

Calcineurin activity in cardiac myocytes (mean + SE, n = 6) increased after 16-h incubation with lipopolysaccharide (LPS) (10 ng/ml) and/or angiotensin II (Ang II) (100 nmol/l) without cumulative effects ($p < 0.05$). This was blocked by pre-treating myocytes with losartan (1 μmol/l, Ang II type 1 receptor inhibitor) 1 h before LPS or Ang II, while losartan alone had no effect.

without 1-h pre-treatment with ryanodine (1 μmol/l, 1 h before LPS), which locks the SR calcium release channel in an open state (32). Figure 3B shows that ryanodine inhibited LPS-induced increase in calcineurin activity ($p < 0.05$, 1-way repeated measures ANOVA, n = 6).

Figure 3B shows that pre-treatment of myocytes with nicotine (15 ng/ml, 4 h before LPS), which inhibits LPS-induced apoptosis (27), prevented increased calcineurin activity with LPS ($p < 0.005$, n = 6).

LPS activation of calcineurin is associated with apoptosis. To determine if calcineurin induces or inhibits cardiac apoptosis with LPS, cardiac myocytes were incubated for 24 h with or without LPS (10 ng/ml) and the calcineurin inhibitor cyclosporin A (0.5 μmol/l, 1 h before LPS). We previously observed peak changes in TUNEL staining to occur 24 h after LPS in this model (10). Figure 4A demonstrates that cyclosporin A blocked an LPS-induced increase in TUNEL staining ($p < 0.05$, 2-way repeated measures ANOVA, n = 7). For comparison, a similar protocol was performed in myocytes incubated for 24 h with or without Ang II (100 nmol/l) and cyclosporin A (0.5 μmol/l, 1 h before Ang II). Figure 4B shows that cyclosporin A also blocked increased TUNEL staining with Ang II ($p < 0.05$, n = 9).

Similar results were obtained with other inhibitors of LPS-induced calcineurin activation. Cardiac myocytes were incubated for 24 h with or without LPS (10 ng/ml) or Ang II (100 nmol/l), with or without pre-treatment with BAPTA-AM (0.1 μmol/l, 1 h before LPS or Ang II). In Figure 5A, BAPTA-AM prevented increased TUNEL staining with LPS or Ang II ($p < 0.05$, 1-way repeated measures ANOVA, n = 5). In a separate protocol, cardiac myocytes were incubated for 24 h with or without LPS (10 ng/ml) or Ang II (100 nmol/l), with or without pre-treatment with thapsigargin (1 μmol/l, 1 h before LPS or Ang II). In Figure 5B, thapsigargin blocked increased TUNEL staining with LPS or Ang II ($p < 0.05$, 1-way repeated measures ANOVA, n = 9). Thus, interventions that inhibited LPS-induced calcineurin activation, including cyclosporin A, BAPTA-AM, thapsigargin, losartan, and nicotine, also prevented LPS and Ang II induced apoptosis. This supports a proapoptotic effect of calcineurin activated by LPS.

LPS-induced left ventricular apoptosis in vivo. The time course for LPS-induced apoptosis was measured in vivo. Figure 6 shows caspase-3 activity in the left ventricle (mean ± SE) immediately (time = 0, n = 18), 2 h (n = 11), 4 h (n = 11), 6 h (n = 7), 8 h (n = 11), and 24 h (n = 19) after injecting LPS (1 mg/kg intravenously) in vivo. Caspase-3

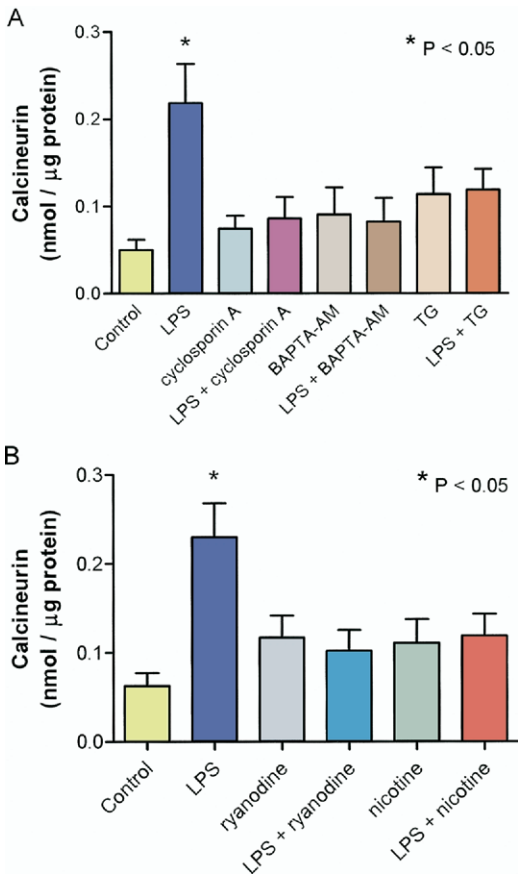

Figure 3 **Cyclosporin A, BAPTA-AM, TG, Ryanodine, or Nicotine Inhibit LPS-Induced Calcineurin Activity**

Calcineurin activity in cardiac myocytes (mean + SE) increased after 16-h incubation with lipopolysaccharide (LPS) (10 ng/ml) compared with control (vehicle) ($p < 0.05$). **(A)** This was blocked by pre-treating myocytes (1 h before LPS) with either cyclosporin A (0.5 μmol/l), 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM) (0.1 μmol/l), or thapsigargin (TG) (1 μmol/l) (n = 5). **(B)** Lipopolysaccharide effects were blocked by pre-treating myocytes with ryanodine (1 μmol/l, 1 h before LPS), or nicotine (15 ng/ml, 4 h before LPS) (n = 6).

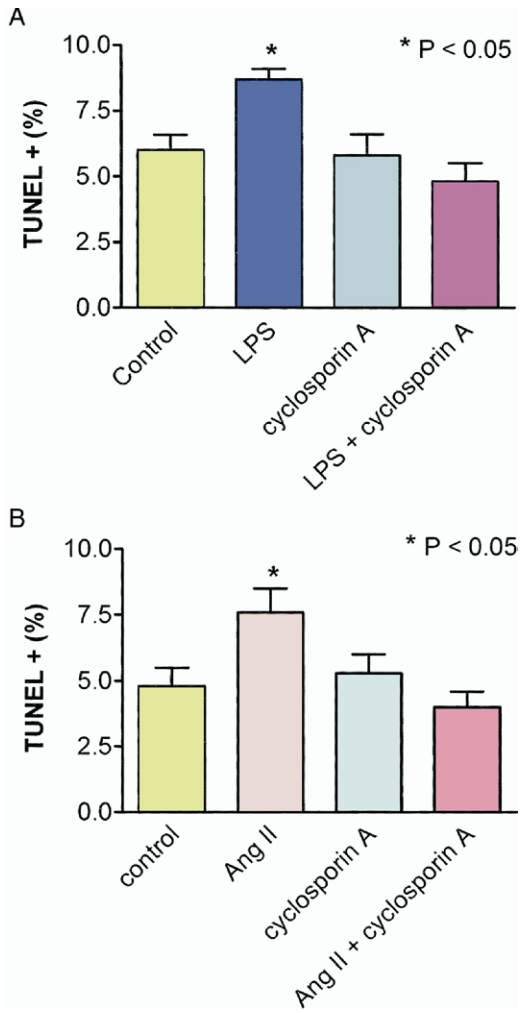

Figure 4 LPS- or Ang II-Induced TUNEL Staining in Cardiac Myocytes Inhibited by Cyclosporin A

Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of cardiac myocytes (mean % + SE) increased after 24 h with lipopolysaccharide (LPS) (10 ng/ml, n = 7) **(A)** or angiotensin II (Ang II) (100 nmol/l, n = 9) **(B)** compared with control (vehicle) ($p < 0.05$). In both cases, this was blocked by the cyclosporin A (0.5 μmol/l, 1 h before LPS or Ang II), while cyclosporin A alone had no effect ($p < 0.05$).

activity increased from 4 to 24 h after LPS ($p < 0.05$ vs. time 0, 1-way ANOVA). This is consistent with the time course for LPS to increase calcineurin activity in cardiac myocytes in vitro (Fig. 1A).

The role of calcineurin in vivo was examined by treating rats with cyclosporin A (20 mg/kg/day subcutaneously) for 3 days before injecting LPS (1 mg/kg) by tail vein. This dose of LPS induces cardiac apoptosis without causing distress or affecting blood pressure (10,27). Figure 7 shows TUNEL staining in the left ventricle in 4 groups of rats (n = 5 per group) 24 h after intravenous injections of: 1) saline (control); 2) LPS; 3) saline after 3 days pre-treatment with cyclosporin A (cyclosporin A); or 4) LPS after 3 days pre-treatment with cyclosporin A (LPS + cyclosporin A). Injecting LPS increased TUNEL staining compared with control, but not in rats pre-treated with cyclosporin A ($p < 0.05$, 2-way ANOVA, n = 5).

Discussion

The major finding in this study is that LPS activates calcineurin in cardiac myocytes, and in this setting, calcineurin has proapoptotic effects. Cardiac calcineurin was activated within hours (t 1/2 = 5 h) by low levels of LPS (EC_{50} = 0.8 ng/ml) that are relevant for subacute and chronic conditions. This was associated with apoptosis that was blocked by the calcineurin inhibitor cyclosporin A in cardiac myocytes in vitro, as well as in the left ventricle in vivo. Calcineurin

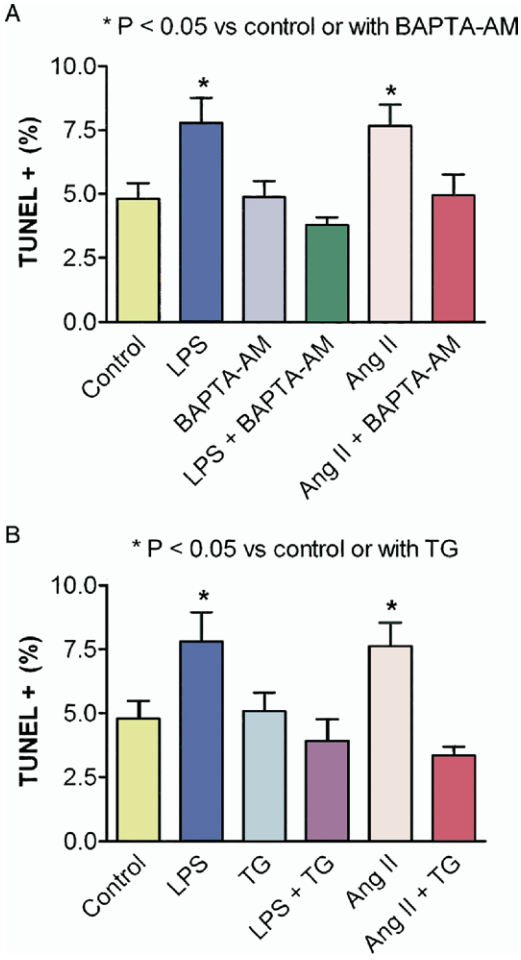

Figure 5 LPS- or Ang II-Induced TUNEL Staining of Cardiac Myocytes Inhibited by BAPTA-AM or Thapsigargin

Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining of cardiac myocytes (mean % + SE) increased after 24 h with lipopolysaccharide (LPS) (10 ng/ml) or angiotensin II (Ang II) (100 nmol/l) compared with control (vehicle) ($p < 0.05$). This was blocked by pre-treating myocytes 1 h before LPS or Ang II with: **(A)** 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM) (0.1 μmol/l), while BAPTA-AM alone had no effect (n = 5); **(B)** Thapsigargin (TG) (1 μmol/l), while TG alone had no effect (n = 9).

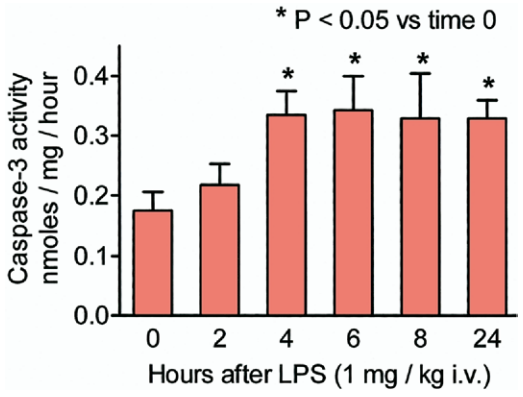

Figure 6 **Time Course for Caspase-3 Activity in Left Ventricle After LPS In Vivo**

Caspase-3 activity in the left ventricle increased from control (time = 0) 4 to 24 h after injecting lipopolysaccharide (LPS) (1 mg/kg intravenously) in vivo ($p < 0.05$, mean + SE, each bar n = 7 to 19).

activation was inhibited by the calcium chelator BAPTA-AM, SR Ca-ATPase inhibitor thapsigargin and SR RyR inhibitor ryanodine, indicating that the activating calcium for calcineurin is SR-dependent. Calcineurin activation was mimicked by Ang II without cumulative effects, and blocked by losartan (AT_1 receptor inhibitor), similar to the pathway that we demonstrated to mediate LPS-induced apoptosis (10). Calcineurin activation was inhibited by nicotine, which inhibits LPS-induced apoptosis proximal to AT_1 receptor activation (27). The simultaneous inhibition of apoptosis with multiple inhibitors of calcineurin, including cyclosporin A, BAPTA-AM, thapsigargin, losartan, and nicotine indicates that calcineurin has proapoptotic effects in the context of LPS.

Low levels of LPS have direct effects on cardiac myocytes (9,10) mediated through TLR4 (8) that are distinct from the cardiotoxic effects that occur when high levels of LPS activate a cascade of mediators in sepsis (33). Circulating LPS levels in the pg/ml to low ng/ml range occur in decompensated heart failure (12), periodontitis (34), chronic infections (e.g., lung, urinary tract), smoking (13), hemodialysis, cirrhosis, pancreatitis, abdominal and cardiothoracic surgery (11). Thus, LPS may activate calcineurin in a variety of clinical scenarios. Calcineurin has proapoptotic effects with LPS, similar to its role with beta-adrenergic stimulation (22), whereas calcineurin has primarily anti-apoptotic effects in hypertrophy (2) and ischemia-reperfusion (23,24).

Chronic activation of calcineurin by LPS may contribute to the progression of pre-existing heart disease since the heart has a limited capacity to compensate for the loss of cardiac myocytes by apoptosis. In support of this postulate, chronic low levels of apoptosis induce heart failure (35), and transgenic mice that overexpress calcineurin have depressed cardiac function (after an initial hypertrophy phase) with decreased survival (36). Recurrent LPS exposure may occur with episodes of decompensated heart failure (12) or with chronic infections such as periodontitis (34). Chronic exposure to low levels of LPS has systemic effects. In periodontitis, markers of inflammation (37,38) and carotid arterial intima media wall thickness (39,40) increase. The effects of chronic LPS exposure on cardiac myocytes, which express the LPS receptor TLR4, have not been examined.

Cyclosporin A may inhibit apoptosis by mechanisms unrelated to calcineurin inhibition. Cyclosporin A inhibits mitochondrial cytochrome c release and caspase activation 4 h after LPS in an isolated heart model (41). Cyclosporin A inhibits the mitochondrial permeability transition pore (MPT) to attenuate cardiac apoptosis with oxidative stress (H_2O_2) (42). Although cyclosporin A inhibits MPT, several agents with variable effects on MPT inhibited LPS-induced calcineurin activation and apoptosis, including losartan, BAPTA, thapsigargin, and nicotine (27). Losartan and nicotine do not have prominent effects on cardiac MPT, although nicotine at 10- to 100-fold higher doses than used in the current study may inhibit MPT in isolated brain mitochondria (43). Thapsigargin directly induces MPT in cardiac myocytes at doses of 15 μM, which would have proapoptotic effects (44), whereas in the current study, 1 μM thapsigargin inhibited LPS-induced activation of calcineurin and apoptosis. The concordance of calcineurin and apoptotic responses over a wide range of conditions indicates that calcineurin is a unique downstream marker and/or mediator of LPS-induced apoptosis. These results, however, do not prove that calcineurin activation is required for LPS-induced apoptosis.

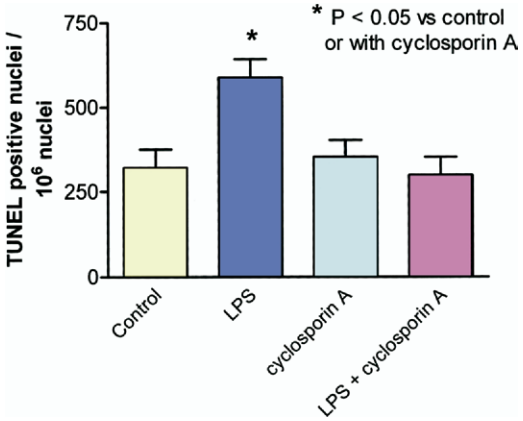

Figure 7 **Left Ventricle Myocyte TUNEL Staining After LPS In Vivo Inhibited by Pre-Treating Rats With Cyclosporin A**

Terminal deoxy-nucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining in the left ventricle (mean + SE, n = 5) increased 24 h after in vivo intravenous injection (tail vein) of lipopolysaccharide (LPS) (1 mg/kg) compared with control (saline injection) ($p < 0.05$, 2-way analysis of variance). This was blocked by pre-treating rats with cyclosporin A (20 mg/kg/day subcutaneously for 3 days before LPS or saline injections), while cyclosporin A treatment alone had no effect.

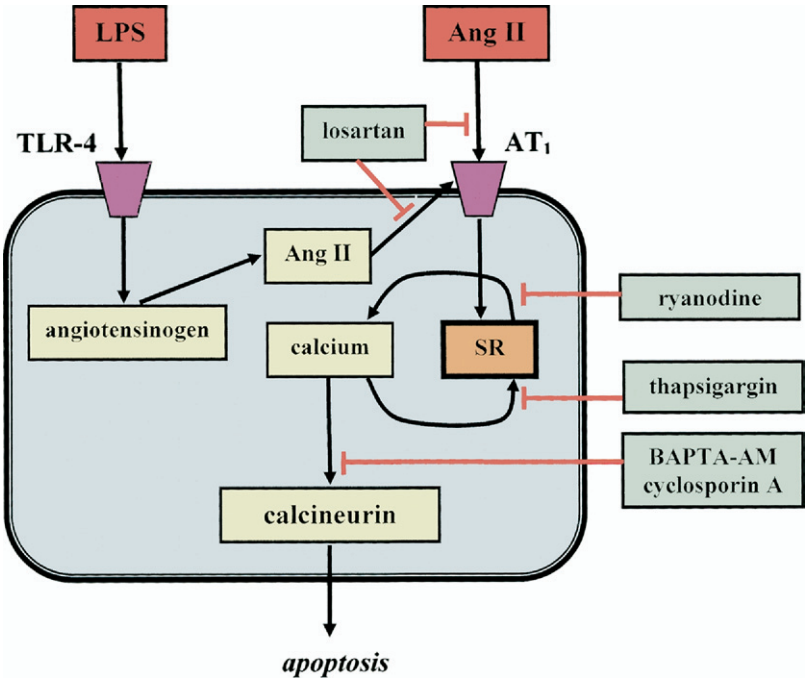

Figure 8 **Proposed Cell Signaling Pathways for LPS-Induced Activation of Cardiac Calcineurin**

Proposed cell signaling pathways for lipopolysaccharide (LPS) induced activation of calcineurin. Inhibitory effects are shown with **red lines**. Ang II = angiotensin II; AT_1 = angiotensin II type 1 receptor; BAPTA-AM = 1,2-Bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester; SR = sarcoplasmic reticulum; TLR-4 = Toll-like receptor-4.

Thapsigargin has other effects besides inhibiting SR Ca-ATPase. Thapsigargin mobilizes calcium from endoplasmic reticulum stores to activate calcineurin and induce apoptosis in neural (45,46) and prostate cancer cells (47). This is unimportant in cardiac myocytes, since thapsigargin inhibits SR Ca-ATPase to attenuate the increase in intracellular calcium with Ang II (48). Furthermore, thapsigargin alone did not affect apoptosis, but inhibited LPS-induced calcineurin activation and apoptosis in cardiac myocytes.

Calcineurin is activated by localized increases in calcium, but the intracellular site has not been experimentally proven (17,18). Calcineurin is activated by low-frequency, sustained elevations in calcium, rather than by high-frequency, high-amplitude transient changes in calcium that occur with each cardiac cycle. Postulated sources for activating calcium include calcium entry through L-type calcium channels, T-type calcium channels, calcium released from the SR, and IP_3R-mediated release of calcium stores (17,18).

The current results identify the source of activator calcium as calcium cycling through the SR. Activation of cardiac calcineurin by LPS or Ang II was prevented by inhibiting SR calcium uptake with the SR Ca-ATPase inhibitor thapsigargin, or by inhibiting SR calcium release with the RyR inhibitor ryanodine. Since the myocytes were non-contractile, calcineurin activation does not require depolarization of L-type calcium channels or the systolic release of calcium from RyR. In non-contractile myocytes, the major source of diastolic SR calcium flux is spontaneous openings of RyR that produce calcium sparks. Since there is a close physical and functional association between calcineurin and RyR (19,20), calcium sparks may activate calcineurin in a localized region at or near the RyR. This is consistent with the concept that cell signaling pathways may be activated by a pool of calcium that is separate from the cytosolic calcium with excitation-contraction coupling (18).

The calcineurin activity with LPS + Ang II combined was less than with Ang II alone (Fig. 2). The cause for this small difference is unknown, but may represent an interaction between LPS and Ang-II-activated pathways. We previously found that LPS activates the inducible nitric oxide synthase (iNOS)-cyclic guanosine monophosphate pathway, which is important for contractile depression (9), but not for apoptosis (10). However, iNOS may play an indirect role by decreasing SR calcium release. In support of this, LPS-activated iNOS depresses beta-adrenergic stimulated ryanodine receptor function (49). A decrease in SR calcium release may result in less calcineurin activation with LPS + Ang II combined, compared with Ang II alone.

The proposed cell signaling pathways involved in LPS activation of calcineurin are summarized in Figure 8 (with inhibitory effects shown with red lines). In the context of LPS, calcineurin has proapoptotic effects.

In conclusion, low levels of LPS activate calcineurin in cardiac myocytes. This involves LPS-induced activation of the cardiac renin-angiotensin system and cardiac AT_1 receptors, and is mediated by SR calcium-dependent mechanisms. This expands the paradigm for cardiac calcineurin, an important mediator of cardiac hypertrophy, to be activated in inflammation and chronic conditions associated with low circulating levels of LPS.

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